

## Real-Time Reverse Transcription-PCR Assay for Detection of Mumps Virus RNA in Clinical Specimens<sup>▽</sup>

Jennifer D. Boddicker,<sup>1</sup> Paul A. Rota,<sup>2</sup> Trisha Kreman,<sup>1</sup> Andrea Wangeman,<sup>1</sup> Louis Lowe,<sup>2</sup> Kimberly B. Hummel,<sup>2</sup> Robert Thompson,<sup>1</sup> William J. Bellini,<sup>2</sup> Michael Pentella,<sup>1</sup> and Lucy E. DesJardin<sup>1\*</sup>

University of Iowa Hygienic Laboratory, University of Iowa, Iowa City, Iowa,<sup>1</sup> and Measles, Mumps, Rubella, and Herpesviruses Laboratory Branch, Centers for Disease Control and Prevention, Atlanta, Georgia<sup>2</sup>

Received 20 March 2007/Returned for modification 4 June 2007/Accepted 16 July 2007

**The mumps virus is a negative-strand RNA virus in the family *Paramyxoviridae*. Mumps infection results in an acute illness with symptoms including fever, headache, and myalgia, followed by swelling of the salivary glands. Complications of mumps can include meningitis, deafness, pancreatitis, orchitis, and first-trimester abortion. Laboratory confirmation of mumps infection can be made by the detection of immunoglobulin M-specific antibodies to mumps virus in acute-phase serum samples, the isolation of mumps virus in cell culture, or by detection of the RNA of the mumps virus by reverse transcription (RT)-PCR. We developed and validated a multiplex real-time RT-PCR assay for rapid mumps diagnosis in a clinical setting. This assay used oligonucleotide primers and a TaqMan probe targeting the mumps SH gene, as well as primers and a probe that targeted the human RNase P gene to assess the presence of PCR inhibitors and as a measure of specimen quality. The test was specific, since it did not amplify a product from near-neighbor viruses, as well as sensitive and accurate. Real-time RT-PCR results showed 100% correlation with results from viral culture, the gold standard for mumps diagnostic testing. Assay efficiency was over 90% and displayed good precision after performing inter- and intraassay replicates. Thus, we have developed and validated a molecular method for rapidly diagnosing mumps infection that may be used to complement existing techniques.**

Mumps infection results in an acute illness with symptoms which often include fever, headache, and myalgia, followed by swelling of the salivary glands. The parotid salivary glands (which are located within the cheek, near the jaw line, and below the ears) are the areas most frequently affected. Complications of mumps can include meningitis, deafness, pancreatitis, orchitis, and first-trimester abortion (14). Vaccines for mumps have been available since the 1960s and, in the United States, the live attenuated Jeryl-Lynn strain is included in the measles, mumps, rubella (MMR) vaccine (6). The MMR vaccine is currently given on a two-dose schedule, and proof of vaccination is required for entry into U.S. schools. One dose of mumps containing vaccine has an efficacy of 88% (8), with efficacy increasing to approximately 95% with two doses (4). Since the MMR vaccine went into routine use in the United States, the incidence of mumps has been reduced by  $\geq 97\%$  (6), with an average of 265 cases reported per year since 2001 (3).

In December 2005, a mumps epidemic occurred in the state of Iowa, with a total of 1,643 confirmed cases and 315 probable cases reported by the Iowa Department of Public Health by September 2006 (2). The mumps epidemic spread to neighboring states, with over 1,000 confirmed and probable cases reported by May 2006 in Illinois, Kansas, Missouri, Nebraska, Pennsylvania, South Dakota, and Wisconsin (3). At present, it is unclear why the epidemic occurred in this highly vaccinated

population. Possibilities include waning immunity in vaccinated individuals and an immune response in vaccinated individuals that was not fully protective against the mumps viral strain that caused the epidemic (1, 15, 18, 20, 21). Investigations to address these possibilities are in progress.

Laboratory confirmation of mumps infection can be made by the detection of immunoglobulin M (IgM) antibodies specific to mumps virus in acute-phase serum samples, by the isolation of mumps virus in cell culture, or by the detection of the mumps virus RNA by reverse transcription (RT)-PCR. In an unvaccinated individual, mumps-specific IgM antibodies do not appear until 3 to 4 days after the onset of symptoms. However, in previously vaccinated individuals who become infected with mumps, the timing and duration of the IgM response are much more variable or nonexistent. In a non-vaccinated individual, mumps virus RNA can be detected in saliva and urine samples for up to 13 days after the onset of symptoms (16, 23, 24).

Mumps virus is a negative-strand RNA virus in the family *Paramyxoviridae*. The mumps virus genome contains seven genes encoding the nucleocapsid (N), phosphoprotein (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins (14). The SH gene, which encodes a protein of 57 amino acids that is thought to block apoptosis in infected cells (25), is the most variable region of the mumps genome and is used to genotype mumps strains (10, 17). In this report, we describe the development and validation of a multiplex real-time RT-PCR test for the detection of RNA from mumps virus in patient specimens, using primers and probes that target the mumps SH gene and the human RNase P (RNP) gene as an internal control.

\* Corresponding author. Mailing address: University of Iowa Hygienic Laboratory, 102 Oakdale Hall, H101-OH, Iowa City, IA 52242. Phone: (319) 335-4339. Fax: (319) 335-4555. E-mail: ldesjard@uiowa.edu.

<sup>▽</sup> Published ahead of print on 25 July 2007.



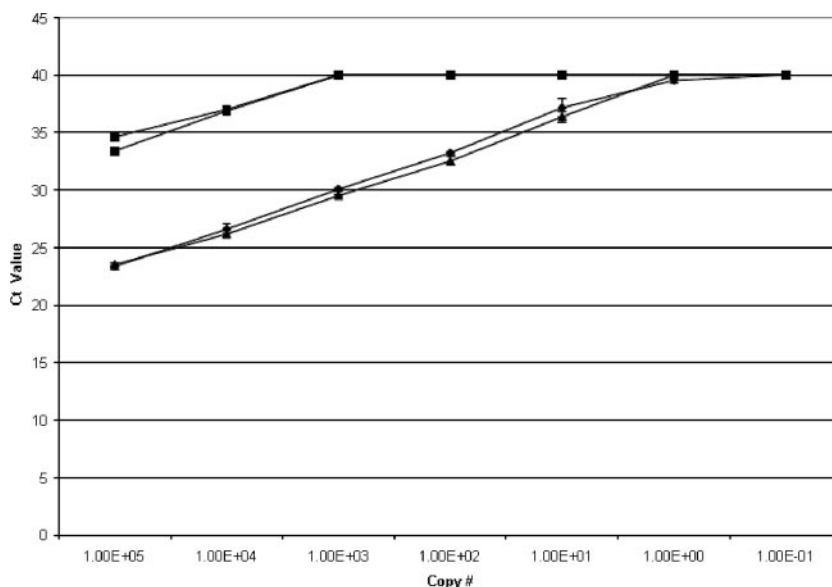


FIG. 2. The lower limit of detection of the RT-PCR assay's ability to amplify SH was determined by amplification of synthetic SH gene RNA. Dilutions (10-fold) of two preparations of synthetic RNA containing the mumps SH were prepared and tested with the real-time RT-PCR assay, either with (triangle and diamond symbols) or without (squares) reverse transcriptase.  $C_T$  values were obtained for each dilution and number of copies of synthetic RNA per reaction.

format thermocycler, an ABI 7500, 7700, and 7000 (Applied Biosystems, Foster City, CA). For the 7500 and 7000 machines, the 9600 emulation was enabled. The primer and probe sets were also tested under the same cycling conditions using reagents within the TaqMan PCR core reagents kit (Applied Biosystems), as follows: 5.5 mM  $MgCl_2$ , 300  $\mu$ M dATP, 300  $\mu$ M dCTP, 300  $\mu$ M dGTP, 600  $\mu$ M dUTP, 0.025 U/ $\mu$ l AmpliTaq Gold, plus 0.25 U/ $\mu$ l MultiScribe reverse transcriptase and 0.4 U/ $\mu$ l RNase inhibitor (Applied Biosystems).

The real-time PCR primers F1073 and R1151 and the probe developed by Uchida et al. were used for the detection of RNA from the mumps F gene (22). Optimization of RT-PCR was performed by various primer and probe concentrations and PCR cycling conditions to obtain a one-step real-time RT-PCR with the TaqMan One-Step RT-PCR Master Mix reagents kit (Applied Biosystems, Foster City, CA) in a 25- $\mu$ l total volume, using 5  $\mu$ l of patient specimen nucleic acid. The following conditions gave the best results and were used for F gene detection (see Fig. 4): 667 nM of each primer and 133 nM of probe; cycling conditions were 48°C for 45 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 59°C for 1 min.

**Interpretation and analysis.** A sample is considered positive by real-time RT-PCR if it crosses the threshold before a cycle threshold ( $C_T$ ) value of 36.5 and is considered equivocal if the  $C_T$  value is  $\geq 36.6$  and  $< 40$ . The multicomponent curve must also reflect that the  $C_T$  value, determined by SDS software, is the result of a true amplification event. A clinical sample is considered negative if the RNP value is  $< 40$  and the SH  $C_T$  value equals 40 or is undetected ("undet"). RNP amplification serves as a positive control that indicates that specimen collection was adequate and that nucleic acid extraction and PCR amplification did occur. Failure to detect RNP in any of the clinical samples may indicate any of the following: improper extraction of nucleic acids from clinical materials resulting in the loss of RNA, the presence of RT-PCR inhibitors in clinical specimens, the absence of sufficient human cells in the sample to enable detection (i.e., poor specimen collection), an improper assay setup and execution, and/or reagent or equipment malfunction. In these cases, the result was reported as "indeterminate." To determine the efficiency of the real-time RT-PCR,  $C_T$  values from a series of 10-fold dilutions of template nucleic acid were plotted on the y axis versus the log of the dilution on the x axis, and the slope of this line was used in the efficiency (E) equation  $E = 10^{(-1/\text{slope})}$ .

**Sequencing the mumps SH gene.** The mumps SH gene was sequenced and analyzed using a protocol modified from that of Palacios et al. (17). Nucleic acids were extracted from mumps shell vial cultures of 34 patient specimens. The SH gene was amplified with a TaqMan One-Step RT-PCR Master Mix reagents kit (Applied Biosystems, Foster City, CA) and primers mumps SH-2S and mumps SH-2AS (Table 1) (17). Cycling conditions were 42°C for 45 min and 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 68°C for 1 min.

Amplified product was purified directly from the PCR using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). Primer SH-1S was used to sequence purified PCR product at the University of Iowa DNA facility. The sequence was analyzed using the mumps virus database found at <http://www.greeneidlab.columbia.edu>. This site performs global alignments based on a comprehensive mumps virus database with the Needleman-Wunsch algorithm to assign genotypes to mumps virus isolates based on the SH gene sequence (17).

## RESULTS

**Specificity.** The specificity of primers and probe for mumps virus used in this protocol was evaluated in silico by performing a BLAST search at <http://www.ncbi.nlm.nih.gov>, and no significant matches were found to DNA sequences other than that for the mumps virus. Specificity was determined empirically by testing nucleic acids that were extracted from cell cultures infected with a variety of agents, including three different genotypes (A, D, and G) of mumps virus, mumps culture-positive patient specimens, mumps culture-negative patient specimens, other human paramyxoviruses, and other respiratory viruses that could be isolated from similar specimen types. We observed positive results from mumps strains A, D, and G. However, no product was detected from cultures of parainfluenza virus type 1 (four isolates), parainfluenza virus type 3 (eight isolates), RSV (four isolates), measles virus, influenza A and influenza B virus, adenovirus, HSV-1, and enterovirus strain Coxsackievirus A9.

The real-time RT-PCR assay detected mumps virus in 34 of 34 buccal/oral swab original specimens that had previously tested positive by viral culture. No positive PCR results were obtained from 40 mumps culture-negative specimens, and the RNP internal control was detected in all specimens. DNA sequence analysis of the SH gene from 31 PCR-positive specimens determined that each was mumps genotype G and that all were identical in sequence except for a G/T-variable nucle-

TABLE 2. Limits of detection<sup>a</sup>

Dilution	<i>C<sub>T</sub></i> value for patient swabs						<i>C<sub>T</sub></i> value for control strain			
	Patient A		Patient B		Patient C		Jeryl-Lynn		Mumps D	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0	19.9	19.49	21.02	21.12	28.32	28.4	24.61	25.48	20.48	20.34
10 <sup>-1</sup>	22.97	22.82	24.46	24.31	31.58	31.34	28.31	29.85	23.66	24.01
10 <sup>-2</sup>	26.49	25.9	27.72	28.25	<b>34.1</b>	<b>34.15</b>	32.09	33.64	27.09	27.24
10 <sup>-3</sup>	29.56	29.46	32.02	31.4	38.19	Undet	<b>35.25</b>	<b>35.8</b>	31.3	31.49
10 <sup>-4</sup>	<b>32.97</b>	<b>32.65</b>	<b>35.29</b>	<b>35.09</b>	Undet	Undet	36.98	Undet	<b>36.37</b>	<b>33.81</b>
10 <sup>-5</sup>	35.91	Undet	Undet	37.23	Undet	Undet	Undet	Undet	Undet	Undet
10 <sup>-6</sup>	37.29	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet
10 <sup>-7</sup>	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet
10 <sup>-8</sup>	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet

<sup>a</sup> A, B, and C represent individual patient buccal swabs that were placed into Vero cell shell vial cultures. Total nucleic acid was extracted from the supernatants following incubation for 5 days. The *C<sub>T</sub>* value for each 10-fold dilution of replicate (Rep) test is recorded. *C<sub>T</sub>* values in bold type represent robust limits of detection for a positive result. Undet, undetected. This value would correspond to the limit of quantification if the assay was performed with a standard curve. Positive results beyond the limit of quantification represent the equivocal range of the assay, where results do not necessarily repeat due to well-known stochastic sampling events.

otide at position 86 in the coding sequence of the gene (GenBank accession number DQ661745). Nucleic acids from mumps vaccine strain A (Jeryl-Lynn) and mumps genotype strain D were sequenced as controls and, as predicted, were determined to be genotypes A and D, respectively.

**Sensitivity and efficiency.** Assay sensitivity was assessed by determining the limit of detection from a dilution series of nucleic acid extracted from cell cultures infected with buccal swab specimens from three different patients and two control strains (Table 2). We found that mumps virus could be detected in dilutions ranging from 10- to 10,000-fold. The test equivalence range appears to be a *C<sub>T</sub>* value of approximately 36. Results for dilutions beyond a *C<sub>T</sub>* value of 36 for each sample were not reproducible, most likely due to well-recognized stochastic properties of PCR with highly diluted nucleic acids. The data presented in Table 2 were used to determine the efficiency of the real-time RT-PCR amplification as described in Materials and Methods. The amplification efficiency and coefficient of variation (CV) were 93.3% ± 2.9% and 3.1%, respectively, for mumps strain D; 96.3% ± 6.8% and 7.1%, respectively, for strain A (Jeryl-Lynn); and 105.3% ± 14.345% and 13.6%, respectively, for strain G. In addition, we determined the efficiency of RNP amplification from a log dilution series of RNA extracted from two original patient specimens to be ~ 83% (data not shown).

The real-time assay targeting the mumps SH gene could reliably detect as few as 10 copies of synthetic SH RNA at a *C<sub>T</sub>* of 36 (Fig. 2). At the rate of one copy of SH RNA per reaction, some of the replicates had *C<sub>T</sub>* values in the equivocal range, and two of four replicates had *C<sub>T</sub>* values >40. All replicates were negative (*C<sub>T</sub>* of >40) at 0.1 copy of RNA. The amplification was linear over a 5-log range in RNA concentrations (Fig. 2) ( $r^2 = 0.994$ ; slope, -3.27), and the calculated efficiency was 102%.

**Precision.** Precision of the assay was determined by performing replicate testing with 34 patient specimens that initially tested positive by RT-PCR, with positive results for each of these specimens confirmed by viral culture. The results from three separate PCR assays performed by different technologists with two different ABI 7500 PCR machines using two lots of reagents are presented in Fig. 3. The results range from a *C<sub>T</sub>*

of 26.35 to 36.03. For all of the specimens tested, the CV value (standard deviation/mean) was below 10%. The average CV for 34 patient samples was 3.4% ± 1.9% for SH and 2.2% ± 1.2% for RNP, suggesting a high level of assay robustness. The interassay variation was also much greater than the intraassay variation, as might be expected.

This assay was developed on an ABI 7500 platform. However, it was also tested on an ABI 7000 and an ABI 7700. A comparison of *C<sub>T</sub>* values obtained from the ABI 7500, ABI 7700, and ABI 7700 performed with nucleic acids extracted from 12 patient specimens, using the same PCR master mix, template RNA, and PCR conditions, demonstrated that these platforms varied in performance. ABI Prism 7700 was the most sensitive instrument and had values that were, on average, ~2 *C<sub>T</sub>* values lower than those with the ABI 7500. The ABI 7000 had ~3 *C<sub>T</sub>* values higher than those of the ABI 7500, resulting in one specimen with a *C<sub>T</sub>* value in the equivocal range on the 7000 compared to that on the ABI 7500 (data not shown).

The protocol for nucleic acid extraction was also performed using the automated MagNA Pure LC Instrument (Roche Diagnostics Corp., Indianapolis, IN), yielding *C<sub>T</sub>* values (within 1 *C<sub>T</sub>* value) comparable to those obtained with manual QIAGEN extractions (data not shown). Similar RT-PCR results were also obtained using a different PCR master mix setup, the TaqMan PCR Core reagents kit with Multiscribe reverse transcriptase (data not shown) (Applied Biosystems).

**Comparison to the F gene real-time assay.** We compared the RT-PCR assay described here to a previously published real-time assay performed by Uchida and colleagues that targeted the F gene of mumps virus in a two-step real-time RT-PCR (22). We chose the method of Uchida et al. for comparative studies because, at the time we initiated this study, it was the only published real-time RT-PCR assay that had been tested with patient specimens. We modified the protocol to use the primer and probe set in a one-step assay. In our hands, this target and protocol were not as sensitive as the SH RT-PCR test reported here, because it did not detect mumps virus from two of 11 patient specimens that were confirmed as culture positive and had higher *C<sub>T</sub>* values overall when tested by using a variety of dilutions of mumps shell vial cultures from patient specimens (Fig. 4). Attempts to increase the sensitivity of the

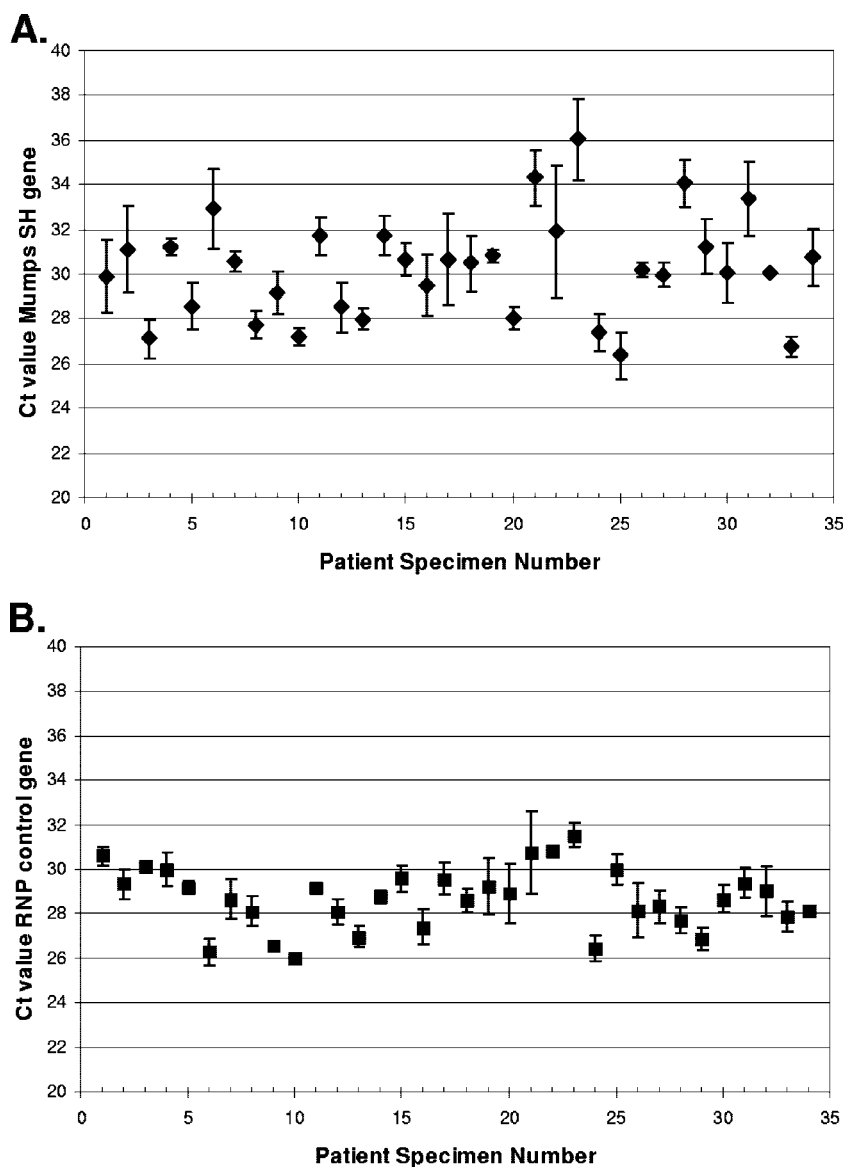


FIG. 3. SH and RNP multiplex amplifications of 34 patient buccal swab specimens. (A)  $C_T$  values obtained from patient buccal swab specimens, using the SH primers and probe. (B)  $C_T$  values obtained from the same patient specimens, using the RNase P primers and probe. Values shown are the means  $\pm$  standard deviations from three experiments that were performed by different technologists on different days.

assay by adjusting primer and probe concentrations were not successful (data not shown). We chose not to test other published PCR protocols for mumps detection because they did not utilize one-step, real-time RT-PCR (11–13, 17, 19).

## DISCUSSION

In this study, we validated a real-time RT-PCR assay for the detection of RNA from mumps virus, using samples obtained during a mumps epidemic in the Midwestern United States in 2006. Our goal was to develop a one-step, real-time RT-PCR test that contained an internal control for nucleic acid extraction and PCR inhibition, as this format would be optimal to apply in an outbreak situation. We found that the test was specific and showed a 100% correlation with results from viral

culture. All specimens that have been detected by RT-PCR were confirmed to be positive by viral culture. This suggests a high degree of accuracy for the molecular test, since viral culture is considered to be the gold standard for mumps diagnostic testing.

RT-PCR results in the equivocal range could not be confirmed by viral culture. The reasons for this could be a very low viral load or nonviable viral particles in the original specimen or a false-positive result due to contamination or cross-reaction. However, contamination is a less likely explanation, since none of our Quality Control indicators (one negative extraction control per five specimens, negative PCR amplification controls, and environmental wipe testing) indicated contamination was present. Additionally, PCR testing was performed using directional work flow, dedicated equipment, and separa-

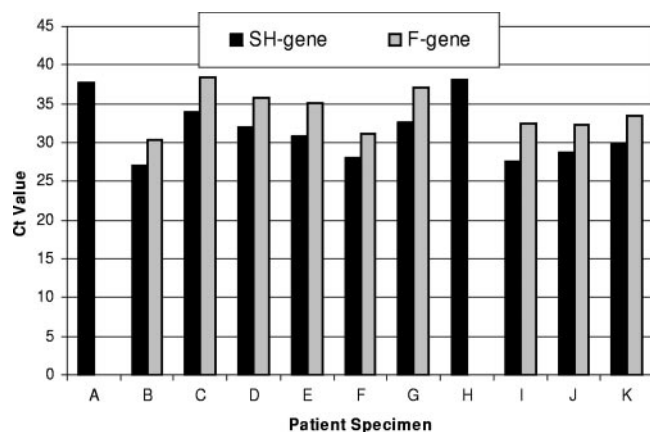


FIG. 4. The RT-PCR assay used to amplify the SH gene is more sensitive than the assay used to amplify the F gene. Eleven patient specimens were tested for the presence of mumps, using SH gene primers or F gene primers. The  $C_T$  values presented are from one experiment that is representative of several experiments utilizing different assay conditions for F gene detection that included modification of primer and probe concentrations and RT and PCR temperatures and times.

tion of PCR activities. The  $C_T$  values ranged from ~26 to 36 for the patient specimens tested, so it is likely that some specimens would have had viral levels in the assay equivocal range. To date, we have tested 1,515 specimens, with only five (0.3%) that fell in the equivocal range (data not shown). The paucity of RT-PCR equivocal results strongly suggests that this test is not significantly more sensitive than viral culture in the diagnosis of mumps infection. It is important to note that the shedding of virus may be less frequent, of shorter duration, or of lower intensity when the infection has occurred in a previously vaccinated individual. In addition, sample collection and transport procedures can affect specimen quality and have an impact on the apparent sensitivity of any assay. The analytic sensitivity of this assay was determined by performing limiting dilution experiments using both mumps culture supernatants and original patient specimens. We could reliably detect mumps virus over a range of 5 logs to a  $C_T$  value of approximately 36, with an assay efficiency of over 90%. This suggests that the sensitivity of the PCR assay is sufficient for the purpose of detecting mumps virus in patient specimens.

We found <10% variation for 34 specimens tested in triplicate, using different technologists and lot numbers of reagents, indicating a high level of precision. Most of the variation came from interassay analysis, with much tighter values observed for intraassay variation, as would be predicted (data not shown). The assay performed well regardless of whether the nucleic acid was extracted by manual or automated methods and with two different sets of PCR reagents. These data suggest that the PCR assay is robust and should be easily transportable.

While this assay is able to detect RNA from mumps viruses in genotypes G, D, and A, detection of the SH gene is variable among strains of wild-type viruses. This variability may result in mismatches between the primer or probe sequence and the target sequence, with the potential for not detecting some mumps strains. However, the SH target proved to be superior

to either the F or N primer and probe sequences in our laboratory (Fig. 4; data for the N gene are not shown). Thus, mumps virus culture along with standard RT-PCR (7) and DNA sequence analysis are still important at the onset of mumps outbreaks. Other PCR diagnostic tests for mumps virus detection have been developed previously, including nested and two-step RT-PCR assays (11, 17, 19, 22). A recent study describes a successful modification of the real-time RT-PCR, used by Uchida and colleagues, into a one-step assay (12). The authors reported a high degree of sensitivity and specificity for oral specimens but not for urine specimens (12). We independently modified the same protocol to a one-step assay but were unable to achieve acceptable sensitivity and specificity. However, the real-time PCR assay validated in this study has a number of advantages, since it is sensitive and specific and utilizes one-step real-time PCR technology to limit the number of manipulations, avoiding the risk of amplicon carryover contamination.

#### ACKNOWLEDGMENTS

We thank Sandra Jirsa, Robin Volk, and Andrea Caldwell for providing excellent technical expertise with virus culture.

#### REFERENCES

- Briss, P. A., L. J. Fehrs, R. A. Parker, P. F. Wright, E. C. Sannella, R. H. Hutcheson, and W. Schaffner. 1994. Sustained transmission of mumps in a highly vaccinated population: assessment of primary vaccine failure and waning vaccine-induced immunity. *J. Infect. Dis.* **169**:77–82.
- Centers for Disease Control and Prevention. 2006. Mumps epidemic—Iowa. *Morb. Mortal. Wkly. Rep.* **55**:366–368.
- Centers for Disease Control and Prevention. 2006. Update: multistate outbreak of mumps—United States, January 1–May 2, 2006. *Morb. Mortal. Wkly. Rep.* **55**:559–563.
- Cohen, C., J. White, E. Savage, J. Glynn, Y. Choi, N. Andrews, D. Brown, and M. Ramsay. 2007. Vaccine effectiveness estimates, 2004–2005 mumps outbreak, England. *Emerg. Infect. Dis.* **13**:12–17.
- Emery, S. L., D. D. Erdman, M. D. Bowen, B. R. Newton, J. M. Winchell, R. F. Meyer, S. Tong, B. T. Cook, B. P. Holloway, K. A. McCaustland, P. A. Rota, B. Bankamp, L. E. Lowe, T. G. Ksiazek, W. J. Bellini, and L. J. Anderson. 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. *Emerg. Infect. Dis.* **10**:311–316.
- Galazka, A. M., S. E. Robertson, and A. Kraigher. 1999. Mumps and mumps vaccine: a global review. *Bull. W. H. O.* **77**:3–14.
- Germann, D., M. Gorgievski, A. Strohle, and L. Matter. 1998. Detection of mumps virus in clinical specimens by rapid centrifugation culture and conventional tube cell culture. *J. Virol. Methods* **73**:59–64.
- Harling, R., J. M. White, M. E. Ramsay, K. F. Macsween, and C. van den Bosch. 2005. The effectiveness of the mumps component of the MMR vaccine: a case control study. *Vaccine* **23**:4070–4074.
- Hummel, K. B., L. Lowe, W. J. Bellini, and P. A. Rota. 2006. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. *J. Virol. Methods* **132**:166–173.
- Jin, L., S. Beard, and D. W. Brown. 1999. Genetic heterogeneity of mumps virus in the United Kingdom: identification of two new genotypes. *J. Infect. Dis.* **180**:829–833.
- Kashiwagi, Y., H. Kawashima, K. Takekuma, A. Hoshika, T. Mori, and T. Nakayama. 1997. Detection of mumps virus genome directly from clinical samples and a simple method for genetic differentiation of the Hoshino vaccine strain from wild strains of mumps virus. *J. Med. Virol.* **52**:195–199.
- Krause, C. H., K. Eastick, and M. M. Ogilvie. 2006. Real-time PCR for mumps diagnosis on clinical specimens: comparison with results of conventional methods of virus detection and nested PCR. *J. Clin. Virol.* **37**:184–189.
- Kubar, A., M. Yapar, B. Besirbellioglu, I. Y. Avci, and C. Guney. 2004. Rapid and quantitative detection of mumps virus RNA by one-step real-time RT-PCR. *Diagn. Microbiol. Infect. Dis.* **49**:83–88.
- Muhlemann, K. 2004. The molecular epidemiology of mumps virus. *Infect. Genet. Evol.* **4**:215–219.
- Nojd, J., T. Teclé, A. Samuelsson, and C. Orvell. 2001. Mumps virus neutralizing antibodies do not protect against reinfection with a heterologous mumps virus genotype. *Vaccine* **19**:1727–1731.
- Okafuji, T., N. Yoshida, M. Fujino, Y. Motegi, T. Ihara, Y. Ota, T. Notomi, and T. Nakayama. 2005. Rapid diagnostic method for detection of mumps virus genome by loop-mediated isothermal amplification. *J. Clin. Microbiol.* **43**:1625–1631.

17. Palacios, G., O. Jabado, D. Cisterna, F. de Ory, N. Renwick, J. E. Echevarria, A. Castellanos, M. Mosquera, M. C. Freire, R. H. Campos, and W. I. Lipkin. 2005. Molecular identification of mumps virus genotypes from clinical samples: standardized method of analysis. *J. Clin. Microbiol.* **43**:1869–1878.
18. Paunio, M., K. Hedman, I. Davidkin, M. Valle, O. P. Heinonen, P. Leinikki, A. Salmi, and H. Peltola. 2000. Secondary measles vaccine failures identified by measurement of IgG avidity: high occurrence among teenagers vaccinated at a young age. *Epidemiol. Infect.* **124**:263–271.
19. Poggio, G. P., C. Rodriguez, D. Cisterna, M. C. Freire, and J. Cello. 2000. Nested PCR for rapid detection of mumps virus in cerebrospinal fluid from patients with neurological diseases. *J. Clin. Microbiol.* **38**:274–278.
20. Rubin, S., J. Mauldin, K. Chumakov, J. Vanderzanden, R. Iskow, and K. Carbone. 2006. Serological and phylogenetic evidence of monotypic immune responses to different mumps virus strains. *Vaccine* **24**:2662–2668.
21. Sanz-Moreno, J. C., A. Limia-Sanchez, L. Garcia-Comas, M. M. Mosquera-Gutierrez, J. E. Echevarria-Mayo, A. Castellanos-Nadal, and F. de Ory-Manchon. 2005. Detection of secondary mumps vaccine failure by means of avidity testing for specific immunoglobulin G. *Vaccine* **23**:4921–4925.
22. Uchida, K., M. Shinohara, S. Shimada, Y. Segawa, R. Doi, A. Gotoh, and R. Hondo. 2005. Rapid and sensitive detection of mumps virus RNA directly from clinical samples by real-time PCR. *J. Med. Virol.* **75**:470–474.
23. Utz, J. P., V. N. Houk, and D. W. Alling. 1964. Clinical and laboratory studies of mumps. *N. Engl. J. Med.* **270**:1283–1286.
24. Utz, J. P., J. A. Kasel, H. G. Cramblett, C. F. Szwed, and R. H. Parrott. 1957. Clinical and laboratory studies of mumps. I. Laboratory diagnosis by tissue-culture techniques. *N. Engl. J. Med.* **257**:497–502.
25. Wilson, R. L., S. M. Fuentes, P. Wang, E. C. Taddeo, A. Klatt, A. J. Henderson, and B. He. 2006. Function of small hydrophobic proteins of paramyxovirus. *J. Virol.* **80**:1700–1709.